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THE CHEMICAL COMPOSITION AND MECHANICAL PROPERTIES OF THE HINGE LIGAMENT IN BIVALVE MOLLUSCS[1]

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Members of the molluscan class Bivalvia have the shell as two calcareous valves which articulate dorsally in the cardinal region to form a hinge. In the area of the hinge is a secreted proteinaceous structure, the ligament, which is common in most members of the class. The ligament acts in opposition to the adductor musculature and functions in opening the two portions of the shell. The form of the ligament varies throughout the Bivalvia; however, in this study only two fundamental mechanical arrangements are considered. When the ligament is dorsal to the pivotal axis, the ligament is subject to tensile stress upon contraction of the adductor musculature. Alternatively, the ligament is positioned between two functionally different areas of the hinge. The part of the ligament ventral to the pivotal axis undergoes compression when the adductor musculature contracts, and it is this part of the system which is responsible for the mechanical characteristics of this type of ligament (Trueman, 1949, 1953; Alexander, 1966). The portion of the ligament dorsal to the pivotal axis is a rigid structure which maintains the juxtaposition of the valves. This latter condition is thought to be the most recent (Dall, 1895), and it is the most common form in extant bivalves. In such forms as *Mytilus edulis*, however, there may be considerable extension and contraction of the outer ligament during movement of the valves (Trueman, 1951).

Contraction of the adductor musculature stores energy in the ligament and relaxation of this musculature results in the release of the stored energy and opening of the valves. Some energy is probably lost as heat; however, the major increment is used in opening the shell.

The animals chosen for this study represent three different modes of life among bivalve molluscs. The swimming forms are represented by *Aequipecten irradians* and *Placopecten magellanicus*. Both species swim by rapidly opening and closing the valves. During swimming the rapid closing of the valves by the large, single adductor muscle, expulses water dorsally with a resultant ventrally directed movement of the animal. The "recovery stroke" or opening of the valves is executed by the inner ligament which is compressed during adduction. These animals are capable of about three opening and closing cycles per second (Alexander, 1966). The inner ligament is reminiscent of a small piece of rubber; it is black, apparently amorphous and without growth lamellae (Fig. 1a). This ligament is mounted on an extension of the shell, the chrondrophore. The outer ligament is minute in trans-

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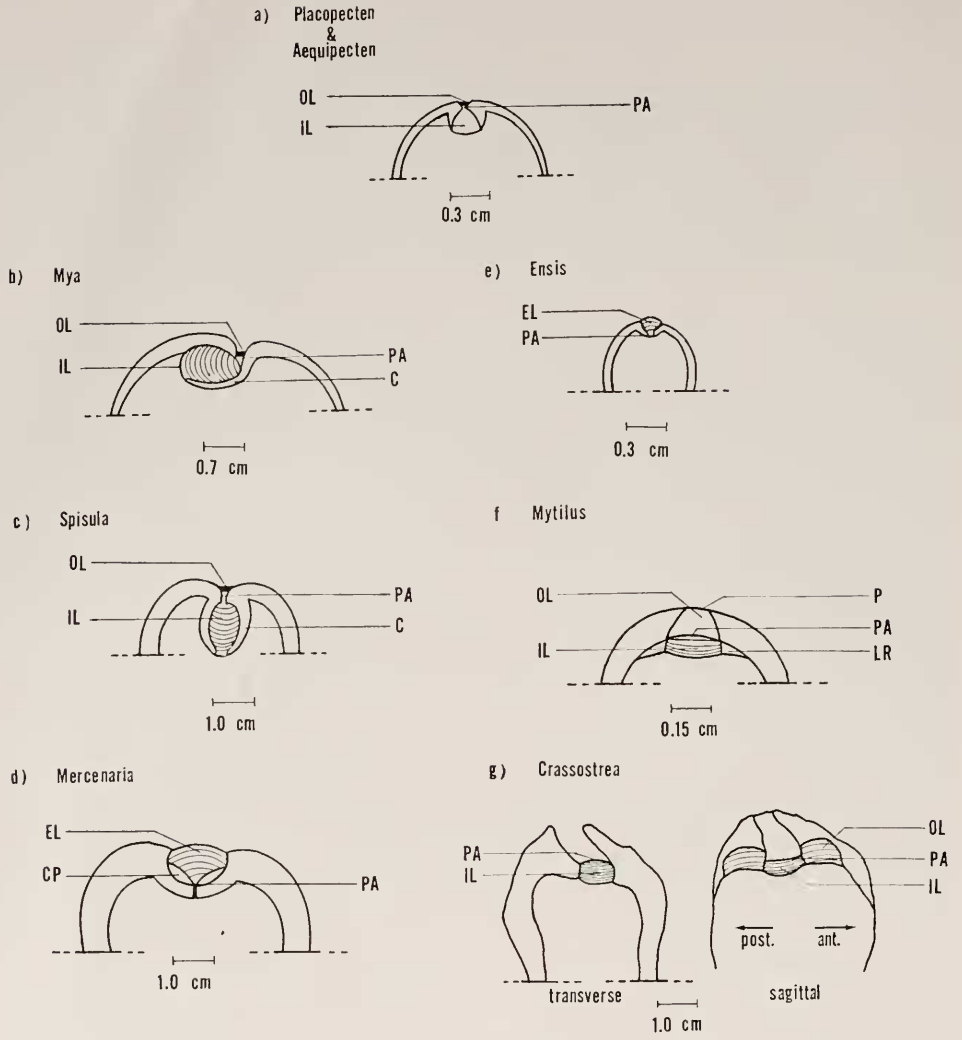

FIGURE 1. Schematic versions of transverse sections through the major ligaments of bivalve molluscs. The growth lamellae visible on the ligaments are indicated where present by repeating lines. These diagrams serve to demonstrate only mechanically functional properties and readers should refer to Owen, Trueman and Yonge (1953) for developmental and morphological details of the ligament. Abbreviations are C, chrondophore; CP, calcified attachment plate; EL, external ligament; IL, inner ligament; LR, ligamental ridge; OL, outer ligament; P, periostracum; and PA, pivotal axis.

verse section; however, it is greatly elongated in the anterio-posterior direction. This portion of ligament contributes little to the mechanical properties of adduction (Trueman, 1949, 1953; Alexander, 1966). The valves of the Pectinidae have no interlocking teeth in the cardinal region of the shell. The elongated outer ligament serves to maintain the juxtaposition of the valves at the point of articulation. *Mya*

arenaria, Spisula solidissima, Mercenaria mercenaria, and *Ensis directus* are representative of the burrowing bivalves. In these species the ligament is not always capable of opening the valves against the force exerted by the mud or sand in which they live; hence, secondary mechanisms may be operative (Trueman, 1954). The ligament of *S. solidissima* (Fig. 1c) may, however, be of sufficient strength to open the shell without aid (Russell-Hunter and Grant, 1962). External ligaments which are thought to be "primitive" (Dall, 1895) are found in *M. mercenaria* and *E. directus* (Fig. 1d and e). Such ligaments are partially under tensile stress when the valves are closed. The other two burrowers display the more "advanced" ligament in which the main body of the ligament has shifted to a position ventral to the pivotal axis of the valves and is thus compressed by the action of the adductors. The ligaments of both *M. arenaria* (Fig. 1b) and *S. solidissima* (Fig. 1c) are mounted on chondrophores. A long chondrophore provides the ligament with a lever to the pivotal axis and, therefore, a mechanical advantage in opening the valves.

Many species of bivalves are sessile or semi-sessile. *Mytilus edulis* and *Crassostrea virginica* (Fig. 1f and g) are characteristic of this mode of life. The resistance to opening the valves encountered by burrowing species is not a problem for attached forms, unless they are living in extremely crowded communities. The gape of the valves of attached species tends to be slight and the rate of opening slow; this is in marked contrast to the Pectinidae.

Growth lamellae are often present in the ligament tissue and represent periods of varying growth potential. The dorsal area of the mantle, the isthmus, is responsible for secretion of the inner ligament. Consequently, the growth lamellae are oriented parallel to the surface of the underlying mantle. The outer layers of the ligament are secreted by the outer lobe of mantle margin (Owen, Trueman and Yonge, 1953).

Most of the publications of the last century which discussed the bivalve ligament were concerned with the phylogenetic relationships (Dall, 1889; Jackson, 1890, 1891); however, Dall (1895) did propose the name "resilium" for the internal ligament in an attempt to indicate its function. Dall (1895) described the function of the primitive ligament as having the essential nature of a C-spring.

The investigations of Trueman (1942, 1949, 1950a, 1950b, 1951, 1953, 1954, 1964, 1966) represent the first and, with few exceptions until this report, the only attempt to approach the study of bivalve ligaments from the aspect of functional morphology. Trueman (1942), in describing the ligament of *Tellina tenuis*, noted birefringence in several parts of the ligament but was unable to deduce its nature. In 1949, Trueman speculated that the birefringence might be due to some substance of lipoid nature. The outermost ligamental layers of *Mytilus edulis* were also reported (Trueman, 1950a) to display birefringence. The staining properties of the ligament can be used to demonstrate that the outer and inner layers of ligament from various bivalves are similar. With Mallory's triple stain the outer layers stain red and the inner layers blue (Trueman, 1951). The ligamental layers and the inner and outer calcareous layers of the shell are derived from the same structure, the mantle, and comparison of the growth lines of the ligament and the corresponding lines in the valves is possible. Both layers of the ligament are considered to be conchiolin (the organic phase of shell) as the ligamental layers represent local modifications of shell layers (Owen *et al.*, 1953; Trueman, 1964). The conchiolin

of *Anodonta* ligament is heavily tanned by orthoquinones in the outer layer, but only slight tanning occurs in the inner layer (Trueman, 1950b). Heavily tanned outer ligaments seem to be of general occurrence. This sclerotization of the outer ligament with chemical bonding of adjacent polypeptide chains enhances the mechanical ability of the ligament to withstand tensile stress (Trueman, 1964). Recently Andersen (1967) has isolated 3,3′-methylene-bistyrosine from whole ligaments of *Mytilus edulis* and the inner ligaments of *Spisula solidissima* and *Pecten maximus*. This compound represents, according to Anderson (1967), a unique method of protein tanning. The two most common elastic proteins are cross-linked with di-tyrosine and tri-tyrosine (resilin from insect cuticle), and desmosine and isodesmosine (elastin, the vertebrate elastic protein). Beedham (1958) conducted an amino acid analysis on the ligament of *Anodonta*, but unfortunately he used visual assessment of areas on paper chromatographs to assay the amount of a particular amino acid in a ligament hydrolyzate. He suggested the relatively high proline content and very low percentage of phenolic amino acids indicated a composition similar to collagen; but the relatively low proportion of glycine, little or no hydroxyproline, and appreciable quantities of methionine were quite unlike collagen. The noncollagenous nature of the ligament was reaffirmed by Hare (1963) who reported no hydroxylysine or hydroxyproline in the ligament of *Mytilus californianus*. However, Hare (1963) found 391 glycine residues per thousand in the outer ligament and 197 in the inner ligament. Both the ligament of *Aequipecten irradians* and *Placopecten magellanicus* were reported to have over 600 glycine residues per thousand (Kelly and Rice, 1967).

Kelly and Rice (1967) further proposed the name "abductin" for the protein of the inner ligament of the "scallop" in order to indicate its function. "Native type" collagen fibrils with an axial repeat generally less than 600 Å have been noted as a minor component of mollusc shell matrix protein (Travis, Francois, Bonar, and Glimcher, 1967). However, unlike ligament, hydroxyproline and hydroxylysine have been reported from mollusc shell matrix protein (Piez, 1961). Only Kelly and Rice (1967) have reported X-ray diffraction patterns for ligament, and they indicate no distinct pattern from the pectens. Galtsoff (1964) published electron micrographs of *Crassostrea virginica* inner ligament (resilum) fixed with 1% osmic acid. A section across the plane of the growth lamellae revealed a honeycomb appearance with holes about 500 Å in diameter. Another section, apparently at right angles to the holes, revealed fibrils varying in diameter from 370 to 500 Å. It is interesting that Travis *et al.* (1967) reported the presence of holes or compartments in sheets of decalcified shell matrix protein. Furthermore, they reported that these holes contained the inorganic crystals and this arrangement was common to all mineralized tissues, both invertebrate and vertebrate thus far studied. Bevelander and Nakahara (1969) maintained that the conchiolin of *Mytilus edulis* and *Pinctada radiata* is homogeneous and not composed of a fibrillar structure.

The organic phase of ligament is often associated with an inorganic phase, $CaCO_3$. Very little research has been directed toward this aspect of ligament morphology. Trueman (1949) reported that treatment with dilute hydrochloric acid indicated the presence of calcareous material in the periphery of the inner ligament of *Tellina tenuis*. Trueman (1964) stated that, in general, outer ligament contains no calcium carbonate and inner ligament typically consists of relatively little

tanned protein, and calcium carbonate. Stenzel (1962) noted that aragonite was the crystalline phase of calcium carbonate in the resilium of *Crassostrea virginica.* Hare (1963) reported aragonite in *Mytilus californianus* ligament, and Bevelander and Nakahara (1969), in a paper concerned with the synthesis of ligament, published electron micrographs of aragonite crystals in the ligament of *Mytilus edulis.*

Trueman (1951) made the first measurements of the physical characteristics of the ligament. He estimated the mass which the valves of *Ostrea edulis* were capable of moving under the influence of the ligament and compared this with the surface area of the valves to determine the relative capability of valve abduction for several bivalve species. The first hysteresis loops for bivalve ligament were published by Trueman (1953) for several species of bivalves. He concluded from this type of data that the outer ligament of *Pecten maximus* probably behaves as a fairly rigid hinge structure. Trueman (1953) also used the percentage difference of the closing and opening moments (very nearly the torque generated by the ligament at closing and the torque generated at opening, respectively) as a measure of the ligament. The difference is about 4% for *Pecten* and about 10 to 20% for other bivalves. Trueman (1953) showed a relatively low internal resistance in *Pecten,* suggesting a more efficient mechanism for the frequent opening and closing of the valves. The unusual efficiency of the ligament of these bivalves is undoubtedly related to the swimming mode of life. Trueman (1953) also stated that the area enclosed by a hysteresis loop is a measure of ligament efficiency, but he did not make this measurement. He did observe, by inspection, that *Pecten* and *Chalmys* ligaments generated hysteresis loops with markedly less enclosed area than most bivalves.

More recent studies on the mechanical properties of ligament were conducted by Russell Hunter and Grant (1962) using *Spisula solidissima.* They concluded that the ligament was the main mechanism opening the shell of this burrowing bivalve. The secondary mechanisms of abduction, mentioned above, were apparently not required in this clam. Alexander (1966) in a lengthy thermodynamic approach to the study of the inner ligament of the Pectinidae concluded that this tissue functions with rubber-like elasticity, in which changes of entropy are more important than changes of internal energy.

The ligament of the bivalve molluscs is thus an unusual structure in that it is a nonliving tissue which carries out a function normally reserved for muscle, *i.e.,* it opposes the action of a flexor muscle. It is the intent of this paper to compare physically and chemically the functional structure of the ligaments of a variety of bivalve molluscs representative of differing life styles.

Materials and Methods

Source and maintenance of experimental animals

The molluscs used in this study, *Placopecten magellanicus* (Gmelin), *Aequipecten irradians* (Lamarck), *Ensis directus* (Conrad), *Mya arenaria* (L.), *Spisula solidissima* (Dillwyn), *Mercenaria mercenaria* (L.), and *Mytilus edulis* (L.), were obtained from the Supply Department of the Marine Biological Laboratory, Woods Hole, Massachusetts. Measurements of the mechanical properties of the ligament of these animals were made at the Marine Biological Laboratory with animals main-

tained in the running seawater system at 18 to 20° C. *Ensis directus* was maintained in trays of sand while the other species were placed on the bottom of the sea table in the running water.

Crassostrea virginica was obtained from commercial fishermen in Kemah, Texas, and maintained in a recirculating artificial seawater (Seven-Seas Marine Mix, Utility Chemical Company) system of 150 gallons capacity until the mechanical properties of the ligament were measured. The artificial sea water was admixed with equal portions of water from the Gulf of Mexico and the salinity adjusted to 33‰ with tap water. The temperature range of the seawater system was 17 to 20° C. No effort was made to retard natural growth of microorganisms or other sources of suspended food in the system. Calcium carbonate and chalk were added to the water to aid in the maintenance of pH and as a supply of calcium ions. The circulating water was passed through glass wool, activated charcoal, and a layer of crushed oyster shell which acted as a filter as well as aerating the system. Several times during the course of the project it was necessary to replenish the supply of animals from the New England area. Live animals were shipped by air freight from the MBL in chilled insulated containers and maintained in the seawater system at Rice University prior to experimentation.

Chemical analysis

Ligament tissue for chemical analysis was carefully dissected away from the valves, rinsed with distilled water, and ground with a mortar and pestle. The powder was dried, *in vacuo*, for 24 hours at 22° C and stored over silica gel at room temperature until preparation for analysis. No chemical change was noted during storage. Fresh ligament tissue was obtained from the animals maintained in the seawater system at Rice University and prepared immediately for analysis.

Amino acids and protein. Amino acid analysis of the ligament tissue was conducted on acid hydrolyzates of the above powder. Samples of 10 to 40 mg were hydrolyzed in 2 ml of 6 N HCl in sealed vials for 24 hours at 120° C (Campbell, 1960). The acid was removed from the hydrolyzate by low temperature vacuum distillation in a "Rotary Evapo-Mix" (Buchler Instruments Co.) and the residues were returned to a known volume in 0.1 N HCl. Since no interference was observed, no attempt was made to remove inorganic ions prior to analysis on an amino acid analyzer. Concentrations of amino acids as low as 10^{-8} M were measurable with this methodology.

A stoichiometric relationship may be assumed between the amide N and the number of asparagine and glutamine residues (Hare, 1963). The amide N was released from the ligament powder by boiling with 2 N HCl (Chibnall, Mangan and Rees, 1958) and the resultant, NH_4Cl, was assayed by the method developed by Seligson and Seligson (1951) as described by Campbell, Bonner and Lee (1968) with the following modification: prior to addition of the K_2CO_3, 0.18 ml of 60% KOH was added to each vial. The addition of this base was necessary because the NH_4Cl was in 2 N acid rather than the 0.1 N acid as described by Campbell, Bonner and Lee (1968). The addition of this base resulted in an increase in the pH to about 1.2, which compared with the pH of the 0.1 N acid used by Campbell *et al.*, and favored the release of ammonia from solution after addition of the K_2CO_3.

Standard solutions containing nitrogen in 2 N acid were made to correspond to the samples. Release of amide N from *S. solidissima* ligament in 2 N HCl at 100° C was complete within 30 minutes. The hydrolysis conditions used for all experiments to measure amide N were 1 ml of 2N HCl per 6 mg or less dried hinge ligament at 95 to 100° C at 1 atm for 1 hour. Recovery of amide N, under these conditions, averaged 87 ± 5.6% and 91 ± 5.9% (s.e.) from authentic samples of asparagine and glutamine, respectively.

Total protein was estimated from Kjeldahl nitrogen assayed by the micromethod of Lang (1958). Recovery of nitrogen from lysine averaged 90 ± 1.6% of the calculated amount in a sample of known concentration. The percentage of nitrogen for each ligament protein was calculated from the amino acid analysis. Total protein was then calculated from the formula:

$$\text{T.P.} = \frac{\text{Per cent total N of ligament (Kjeldahl)}}{\text{Per cent N for ligament protein}} \times 100$$

Carbohydrate. Total carbohydrate was determined on aliquots of the acid hydrolyzate prepared for amino acid analysis using the phenol-H_2SO_4 method of Dubois, Gilles, Hamilton, Rebers and Smith (1956). Variation in sugar contents were determined in a Klett-Summerson colorimeter using a No. 50 Klett filter (470 to 530 mμ).

Lipid. The lipid content of the ligament tissue was estimated by grinding a sample of the dried ligament to a finer powder with a mortar and pestle. This powder was extracted in 2:1 (V/V) chloroform-methanol mixture (Folch, Ascoli, Lees, Meath and LeBaron, 1951) using 5 ml per 30 to 40 mg of ligament powder. The extraction was carried out for 24 hours at 22° C in glass-stoppered test tubes placed on a rotator. After extraction the tubes were cleared by centrifugation, aliquots evaporated, and weighed for lipid content.

Inorganic measurements. Calcium content of ligament tissue was measured by atomic absorption spectrophotometry from aliquots of hydrolyzate prepared for amino acid analysis. To eliminate possible interference by various substances (such as amino acids) the method of standard additions was used (Christian and Feldman, 1970). One per cent lanthanum was included in all standards and samples to reduce flame stable complexes of calcium. All standards and samples contained 0 to 10 micrograms of calcium per ml of 1% lanthanum in 0.6 N HCl. The atomic absorption spectrometer was a "Norelco Unicam, SP90A." All instrument parameters—fuel flow, air flow, slit width, etc.—were those recommended by the manufacturer for the determination of calcium (Willis, 1960).

Crystalline phase of the calcium carbonate was determined by X-ray diffraction. Samples were ground to a fine powder and spread in a thin, even layer on one side of double surface cellulose tape. The clean side was affixed to a petrographic slide to permit attachment to the goniometer head. Intensities of diffracted beams were recorded on a standard wide angle Norelco X-ray diffractometer equipped with an automatic recording device. Nickel filtered copper K_α radiation (35 kV and 18 mA) was used throughout this procedure.

Mechanical properties of ligament

Measurement of hysteresis. Mechanical hysteresis properties of the ligament of each species of bivalve were obtained from animals in which the adductor muscle had been severed and all the tissue teased from the right valve. The experiments were conducted immediately at room temperature. The valves were never permitted to gape more than the maximum observed when the animal was alive. The apparatus used for these measurements is similar to that used by Trueman (1951) and Russell Hunter and Grant (1962). The load (L) was recorded for each angle of gape. In most cases several cycles from fully open (fully open = maximum gape observed in the living animal) to fully closed were estimated. The weight of the right valve (W) and the distance from the centroid of the shell to the center of the hinge (d) were also recorded. The torque, or moment of force about the hinge (M), can be calculated from the formula: $M = d(2L + W)g$, where g is the acceleration of gravity. The constant, 2, reflects the mechanical advantage of the lever system. It has been customary to drop the gravitational factor from the expression (Trueman, 1951, 1953; Russell Hunter and Grant, 1962), which results in $M = d(2L + W)$, where the units are then gram millimeters instead of dyne millimeters.

The angle of gape was calculated from the absolute gape (as measured from the metric scale) and the distance from the ventral lip to the center of the hinge. All calculations and graphs of hysteresis were completed with the aid of a Hewlett-Packard 9100B calculator equipped with an on-line plotter (H. P. 9125B).

Resilience. The term resilience as defined by Alexander (1968) is the work recovered from a material in an elastic recoil, expressed as a percentage of the work previously done in straining it. The resilience is equal to the area under the closing curve in Figure 2 expressed as a percentage of the area under the opening curve. The area in each case represents the product of force and distance, that is, work. Thus

$$\text{Resilience} = \frac{\text{Force} \times \text{Distance (recovered)}}{\text{Force} \times \text{Distance (put in)}} \times 100$$

$$= \frac{\text{Work (recovered)}}{\text{Work (put in)}} \times 100$$

A hypothetical curve for the hysteresis of bivalve ligament is shown in Figure 2. The area under the lower curve as a percentage of the area under the upper curve is used throughout as resilience.

$$\text{Resilience} = \frac{\int (\text{gram-millimeters}) \times d\theta}{\int (\text{gram-millimeters}) \times d\theta} \times 100$$

where θ is the angle of gape. The numerator is the area under the lower curve; the denominator is the area under the upper curve. The distance from the center of the shell to the center of the hinge (in millimeters) is a constant for each animal. The load on the valve (in grams) could be converted to force by multiplying by the acceleration of gravity. Since the load is in both the numerator and denominator this amounts to multiplication by unity. So the only major difference between this

TABLE I

Chemical composition of ligament tissue. Percentages are by weight based on samples of dried powder used for analysis. Standard error of the mean is n = 3, for protein; n = 2, for $CaCO_3$. Symbols are ND, not detected; IL, inner ligament; EL, external ligament.

	Aequipecten (IL)	*Placopecten* (IL)	*Spisula* (IL)	*Mercenaria* (EL)	*Ensis* (EL)	*Mya* (IL)	*Mytilus* (IL)	*Crassostrea* (IL)
% Protein	97.3 ±4.6	98.9 ±2.1	48.8 ±1.1	25.2 ±1.1	25.0 ±1.5	50.5 ±1.4	33.3 ±4.8	33.9 ±1.5
% $CaCO_3$	1.5 ±.06	2.1 ±.19	63.9 ±6.2	86.2 ±7.5	86.5 ±9.9	60.5 ±2.8	64.9 ±3.8	92.0 ±.12
% Carbohydrate	0.83	0.90	0.22	0.13	0.11	0.31	0.36	0.12
% Lipid	ND	ND	ND	ND	ND	ND	ND	ND
% Total	100	102	113	112	112	111	99	126
$\frac{\% CaCO_3}{\% Protein}$	0.015	0.021	1.31	3.42	3.46	1.20	1.95	2.71

definition and that of Alexander (1968) is the use of angular distance. While not strictly analogous to Alexander's definition this approach is sufficient to compare the resilience of several species. This approach was adopted in order to utilize the already accepted method of reporting bivalve ligament hysteresis (see Trueman, 1953, 1945; and Russell Hunter and Grant, 1962).

RESULTS

Protein, calcium carbonate, carbohydrate, and lipid were determined and the results are reported in Table I. Outer ligaments which do not contribute significant thrust to the opening of the valves (Trueman, 1949, 1953; Alexander, 1966) were not considered in the analysis. Carbohydrate was found to contribute less than one per cent of the ligamental tissue and no lipid was detected by the gravimetric method employed. The absence of lipid in this tissue is significant in relation to Trueman's (1949) suggestion that the birefringence he observed in the ligament of *Tellina tenuis* might be of lipoid nature. The ligament of *T. tenuis* was not investigated in this study, however, birefringence of a nonlipoid nature was noted in the ligament of *Spisula solidissima*. The ligament portions of the species investigated were essentially composed of protein and calcium carbonate in differing proportions. The ratio of calcium carbonate/protein varied from 0.015 for *Aequipecten* to 3.46 for *Ensis*.

The per cent calcium carbonate was calculated from the calcium ion concentration determined by atomic absorption spectrophotometry. The presence of calcium carbonate was verified by X-ray diffraction; however, in the case of *Aequipecten* and *Placopecten* ligament the presence of calcium carbonate could not be ascertained by this method. No inorganic reflections were observed with either rapidly scanning, wide angle, X-ray powder technique or a four hour, wide angle, transmission Laue exposure. Thus, there is no evidence that the relatively small calcium ion concentration, determined by atomic absorption, is representative of a calcium carbonate phase in the ligament of these two species. There are three possibilities; the ionic calcium is not from calcium carbonate, the calcium is amorphous calcium

TABLE II

Amino acid analysis of bivalve ligament and shell protein expressed as residues per 1000. A dash indicates below level of detection or not calculated.

	Aequipecten (IL)*	*Placopecten* (IL)*	*Spisula* (IL)	*Spisula* (OL)	*Mercenaria* (EL)	*Ensis* (EL)	*Mya* (IL)	*Mytilus* (IL)	*Crassostrea* (IL)	*Crassostrea*** shell protein (prismatic)
Cystine/2	2.4	3.7	10.3	8.3	20.9	14.7	15.2	12.5	18.4	7.3
Aspartic acid	17.1	60.2	23.2	47.4	115.0	239.3	41.4	38.6	199.7	122.0
Threonine	10.5	5.9	8.0	31.7	13.9	15.9	14.1	14.4	26.9	18.0
Serine	60.2	28.6	21.5	57.9	13.0	19.1	29.4	89.2	49.0	123.0
Proline	11.0	5.9	27.8	51.6	102.0	71.5	55.5	47.3	99.5	70.0
Glutamic acid	13.0	18.3	19.8	36.2	26.4	34.1	18.8	37.4	61.9	33.0
Glycine	683.9	678.5	628.5	481.2	289.7	295.1	532.0	410.5	156.9	337.0
Alanine	29.6	21.6	34.6	22.0	49.3	46.3	61.6	41.6	85.8	80.0
Valine	4.7	2.0	9.3	37.6	21.2	30.0	27.7	22.3	31.0	20.0
Methionine	59.6	88.7	132.0	26.1	230.2	134.5	133.3	96.6	158.9	3.5
Isoleucine	6.4	3.2	21.6	44.2	15.3	7.9	10.8	31.3	20.4	12.0
Leucine	1.9	2.3	18.7	32.3	17.6	35.1	10.1	72.2	16.3	38.0
Tyrosine	1.1	8.4	1.9	42.3	2.5	—	—	3.9	11.3	46.0
Phenylalanine	86.0	51.2	19.9	26.2	8.3	25.8	21.2	22.3	17.5	31.0
Lysine	8.6	12.7	10.3	16.2	42.9	19.7	7.5	34.0	14.1	15.0
Histidine	—	—	2	12.4	5.3	4.3	3.4	4.0	7.5	17.0
Tryptophan	—	—	—	—	—	—	—	—	—	—
Arginine	4.1	8.8	11.0	36.4	26.2	5.7	18.1	22.0	24.9	27.0
Hydroxylysine	—	—	—	—	—	—	—	—	—	—
Hydroxyproline	—	—	—	—	—	—	—	—	—	—
$\frac{\text{Asp} + \text{Glu} - \text{Amide N}}{\text{Lys} + \text{His} + \text{Arg}}$	0.18	1.57	1.24	—	1.60	6.39	1.20	0.98	3.98	—
Amide N	27.8	44.8	14.1	—	22.7	83.3	25.1	16.8	77.0	—

* These results are in essential agreement with Kelly and Rice (1967).

** Travis *et al.* (1967).

carbonate, or the X-ray analysis was not sufficiently sensitive to detect the crystalline phase.

Ligament tissue, like many structural proteins such as elastin, resilin, keratin, and collagen (old), are found in a solid phase which is relatively insoluble. The results of the amino acid analysis are shown in Table II. The outer ligaments were not considered; however, the outer ligament of *Spisula solidissima* was included for comparative purposes, as was the shell protein from *Crassostrea virginica*. The most striking feature of these analyses is the concentration of glycine found in the ligaments of the eight species of bivalves examined. Over 500 glycine residues per thousand total residues were measured in the ligament hydrolyzates of four of the eight species examined. Only *C. virginica* had fewer than 200 glycine residues per thousand, while the presence of nearly 70% glycyl residues occurred in *A. irradians* and *P. magellanicus* hinge ligament protein. Among the ligaments examined, *C. virginica* had the highest concentration of alanine (9%). A significant difference between ligament protein and many other structural proteins is the presence of the sulfur-containing amino acids, methionine and cystine/2. The presence of cystine/2 residues may be indicative of the degree of cross-linking in the ligament protein. It is obviously important that the cross-links be relatively few and widely spaced so that stretching may occur without rupture of strong bonds. Some cross-linking is necessary, however, to prevent the slipping of one chain past another (Weis-Fogh, 1961; Partridge, 1962), yet a considerable degree of cross-linking may be possible without impairing mechanical function. The external ligaments of *Mercenaria* and *Ensis* are subject to stretching during valve adduction. Covalent cross-linking would probably not be incompatible with this functional morphology. The added firmness resulting from cross-linking may, in part, account for the greater strength of the ligaments which contain the greatest amounts of cystine/2.

Proline residues commonly represent 10 to 20% of the total residues of structural proteins (Seifter and Gallop, 1966). The ligament proteins from the *Aequipecten irradians* and *Placopecten magellanicus* are the lowest in proline content of the bivalves examined. The absence of this amino acid could permit considerable hydrogen bonding and rubbery elasticity could be seriously impaired by extensive hydrogen bonding between polypeptide chains. The concentration of valine, phenylalanine, tyrosine and histidine are characteristically low in collagen (Harrington and Von Hippel, 1961) and a similar situation prevails in ligament protein. It should be noted that during acid hydrolysis the concentration of several amino acids may be altered. Tryptophan is completely destroyed while serine, threonine, lysine, arginine, as well as histidine may be partially degraded. The amide nitrogen

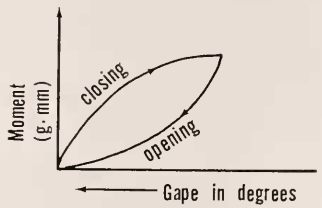

FIGURE 2. Typical mechanical hysteresis.

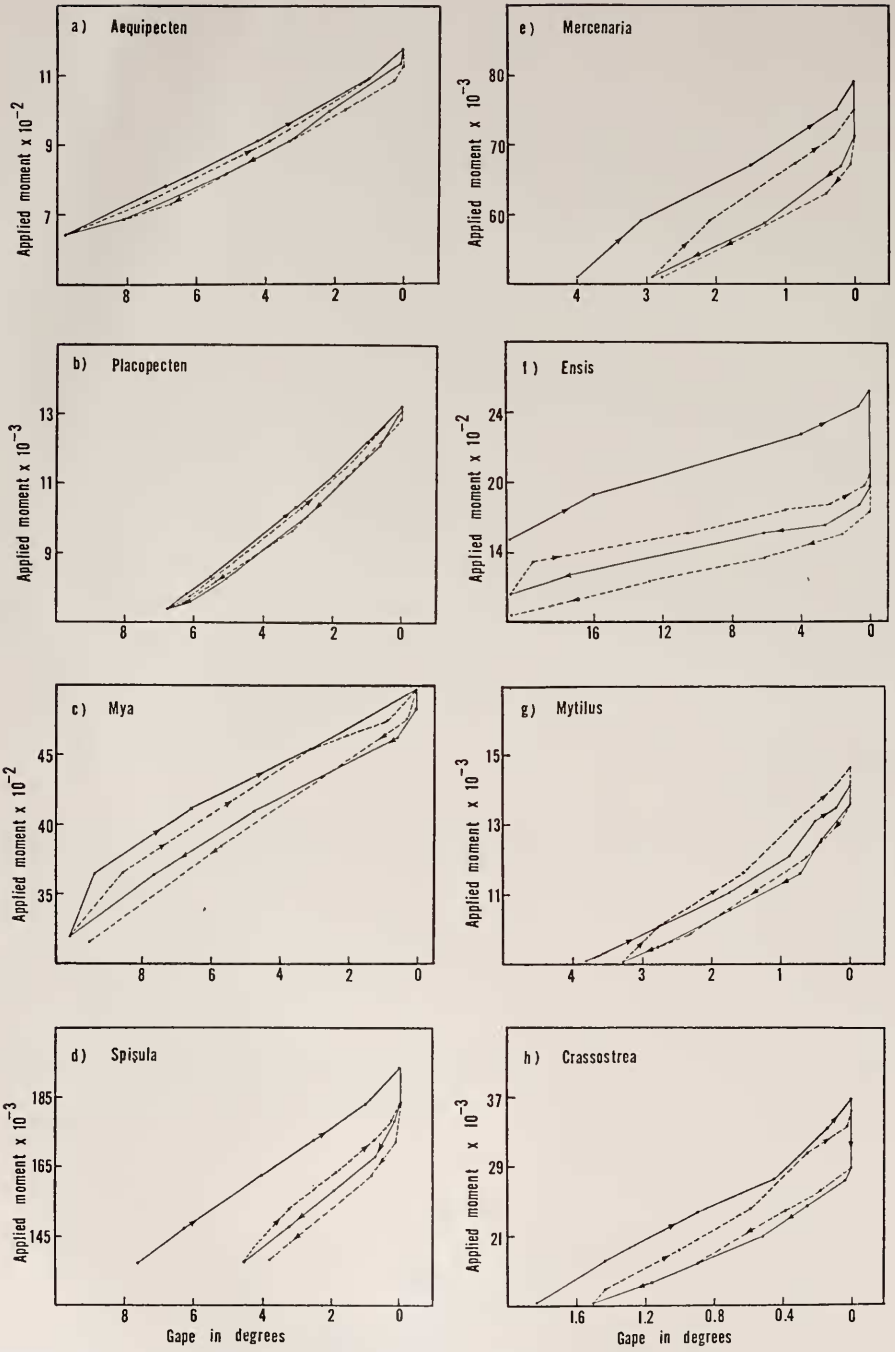

FIGURE 3. Hysteresis curves generated by the hinge ligaments from eight species of bivalve molluscs. The applied moment in g/mm is represented on the ordinate. The angle of gape

of glutamine and asparagine is hydrolyzed resulting in an apparent increase of glutamic and aspartic acids, respectively.

Both elastin and resilin are plasticized with about 50 to 60% water, and become rigid and glasslike when dried (Seifter and Gallop, 1966). Hydrated inner hinge ligament powder from *Spisula solidissima* achieved constant weight when dried *in vacuo* over $CaCl_2$ for 24 hours; the weight loss was $11.95 \pm 0.44\%$. Drying at 98° C and ambient pressure resulted in a further loss of $2.6 \pm 0.1\%$ during the first 24 hours. No significant losses in weight were noted after 48 and 72 hours at 98° C. The ligaments of all eight species became quite hard and brittle upon drying at room temperature, yet the characteristic resilience was restored upon rehydration. Glycerol can plasticize resilin and cause it to swell (Seifter and Gallop, 1966); however, the ligament of *S. solidissima* shrinks markedly and loses its resilience when exposed to glycerol at room temperature for several days. Further, *Spisula* inner ligament was not affected by 0.5 N HCl or NaOH at room temperature, and underwent no visible changes when heated to 100° C in water for 5 to 6 hours, characteristics quite different from collagen.

Ashing *S. solidissima* inner ligament for 20 hours at 450° C resulted in recovery of $56.7 \pm 0.4\%$ of the weight of the dry powder. Atomic absorption analysis indicated $63.9 \pm 6.2\%$ calcium carbonate in the inner hinge ligament (Table I). X-ray diffraction patterns of the ash, by the powder technique, revealed strong aragonite reflections and, occasionally, weak calcite reflections. Calcite reflections never occurred in the diffraction patterns of fresh ligament; therefore, some aragonite may have shifted phase to the more stable calcite configuration during the ashing procedure. The inner hinge ligaments of *Aequipecten irradians* and *Placopecten magellanicus* presented no calcium reflections in the diffraction pattern; however, the remaining six species in this study all contained aragonite as the calcium carbonate phase in that structure.

The mechanical hysteresis of the ligaments of all eight species were examined and representative curves are shown in Figure 3. *Aequipecten irradians* and *Placopecten magellanicus* have the narrowest loops which indicate resilient ligaments while the widest loops, the least resilient ligaments, occur with *Ensis directus*. The curves indicated by broken lines are the second cycle of loading and unloading. The second curve is usually displaced to the right of the first curve. Thus, any particular moment applied to the valves usually results in a greater closure in the second load-unload cycle. Several of the curves do not form closed loops (*e.g.*, *Spisula*). In this situation the valves have failed to achieve the initial gape under the initial load conditions; however, it was observed that the valves of *Spisula* do reset in about 20 minutes.

The resilience of the ligament of each species was calculated from the hysteresis curves and the results are reported in Table III. The ligament resilience of the swimmers, *A. irradians* and *P. magellanicus*, exceeds the other species. The swimming life style required the valves to open and close rapidly; hence, a very resilient ligament is clearly advantageous to this mode of existence. Trueman (1953) reached a similar conclusion about the ligaments of *Pecten* and *Chalmys*.

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of the valves is presented as degrees on the abscissa. The first load-unload curve is represented by the solid line; the second, by the hatched line. Arrows indicate the direction in which the right valve was moving.

TABLE III

Resilience of ligaments, calculated from the areas under the hysteresis curves. n = Number of hysteresis loops for which the mean and standard error were calculated. No more than two loops from any one specimen were used in the calculation.

Species	Mean resilience ± s.e.	*n*
Aequipecten	96.15 ± 0.25	12
Placopecten	96.59 ± 0.36	13
Mya	92.33 ± 0.75	9
Spisula	93.56 ± 0.69	11
Mercenaria	91.93 ± 0.68	12
Ensis	81.81 ± 2.15	8
Mytilus	93.14 ± 1.31	10
Crassostrea	83.94 ± 1.06	11

It is striking that the burrowers (except *Ensis*) have very resilient ligaments. The burrowing life style required that the valves open against the force of a sandy or muddy substrate (Trueman, 1954) which would require a slow, powerful abduction during burrowing (Russell Hunter and Grant, 1962). Trueman (1966) found that even in burrowers which rely heavily on secondary valve abduction mechanisms there is a stage during burrowing which is ligament-dependent and apparently very rapid (Trueman, 1966, stage 5). Furthermore, bivalves are known to clear the mantle of unwanted foreign matter (pseudofaeces) by occasional, rapid opening and closing of the valves.

The opening moment is that value of applied moment which is recorded as the valves just begin to gape during the unloading cycle. Russell Hunter and Grant (1962) found the opening moment of *Spisula solidissima* to be nearly constant for successive cycles of loading and unloading. Opening moments of repeated loading and unloading cycles are not constant in the eight species of bivalves examined in this study. However, the opening moment tends to be the most nearly constant value from any set of cycles; therefore, the opening moment expressed per gram of shell is taken as an indication of the absolute strength of the abduction system (Fig. 4).

DISCUSSION

The principal feature of the molluscan hinge ligament is the high glycine content in the organic phase (Table II). Collagen is characterized by a high glycine content and the presence of 30 to 50% glycyl residues is almost thematic in structural proteins (Seifter and Gallop, 1966). The absence of hydroxyproline and hydroxylysine, the acid residues diagnostic of collagen, alludes to the noncollagenous nature of the bivalve ligament. Alanine, commonly present from 12 to 40% in structural proteins (Seifter and Gallop, 1966), is present in relatively low concentrations in the ligament hydrolyzates. Also significant of the ligament protein is the presence of sulfur containing amino acids. Both methionine and cystine/2 are characteristically very low or absent in collagen (Harrington and Von Hippel, 1961), and are similarly low or absent in elastin and resilin (Seifter and Gallop, 1966). Elastin and resilin are thought to exhibit rubbery elasticity (Weis-Fogh, 1961).

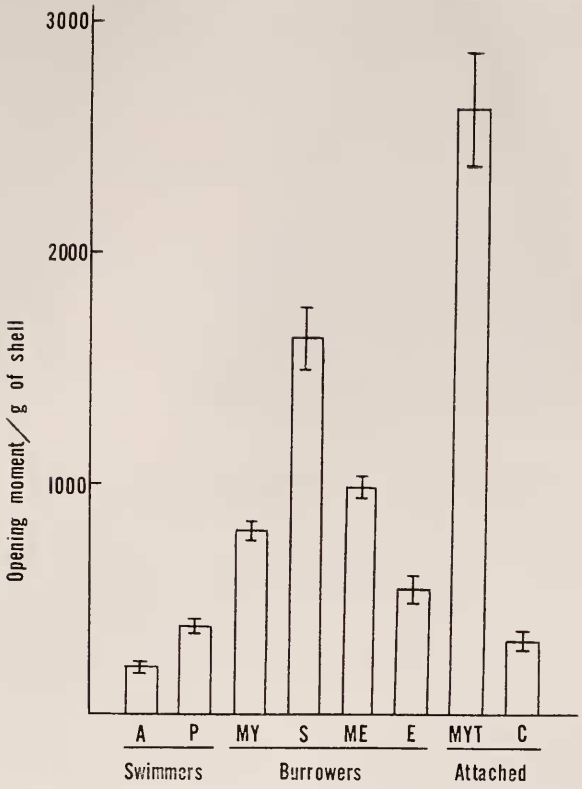

FIGURE 4. Relative strength of the abduction system in some bivalve molluscs. Abbreviations are A, *Aequipectin*; P, *Placopectin*; MY, *Mya*; S, *Spisula*; ME, *Mercenaria*; E, *Ensis*; MYT, *Mytilus*; and C, *Crassostrea*.

About 50 to 60 cystine/2 residues per thousand are found in keratin and are thought to be involved in the cross-linking of protein chains (Seifter and Gallop, 1966).

The pattern suggested by the amino acid analysis of the bivalve ligament is one of large nonpolar regions of protein (Table II). The peptide chains, unlike those of most other structural proteins, except the low glycine in keratin, may be cross-linked with disulfide bonds. It has also been reported that the ligament protein is cross-linked with 3,3′-methylene-bistyrosine (Andersen, 1967). Cross-linking may be associated with the compressible nature of the inner ligament. The presence of extensive amounts of cross-linking in elastin, and perhaps the other structural proteins, would be incompatible with the degree of stretching required of these proteins. Added stiffness, while not compatible with long tensile deformation, would add to the ligament's ability to abduct the valves.

Hydrolyzates from *Aequipecten irradians* and *Placopecten magellanicus*, the two swimming bivalves, tend to be unique with respect to the other species. They contain the greatest concentration of glycine residues, and the least amount of cystine/2 and proline (Table II). Thus, they contain the largest amounts of nonpolar amino acids, probably with very few cross-linkages or hydrogen bonds

TABLE IV

Correlation coefficients for sets of structural and physical parameters.

			Correlation coefficient
$CaCO_3$/protein	*vs.*	resilience	−0.88
$CaCO_3$/protein	*vs.*	$\frac{\text{opening moment}}{\text{g of shell}}$	0.37
Glycine/1000	*vs.*	resilience	0.94
Glycine/1000	*vs.*	$\frac{\text{opening moment}}{\text{g of shell}}$	−0.09
Cystine/2	*vs.*	resilience	−0.86
Cystine/2	*vs.*	$\frac{\text{opening moment}}{\text{g of shell}}$	0.23

between chains. The low proline content assures that the peptide chains will not be prevented (by this amino acid, at any rate) from hydrogen bonding with one another. While the evidence at this point is circumstantial, it does appear that based on comparative amino acid analysis alone, the ligaments of the pectens are most suited to a rubber-like elasticity. Hydrogen bonding, such as could be present in *A. irradians* or *P. magellanicus*, would presumably limit the freedom of movement of the chains and reduce the chances of true rubber-like elasticity. The absence of lipid and the low concentration of carbohydrate are indicative of all the bivalve ligament studies (Table I).

Calcium carbonate commonly occurs in living systems as calcite, aragonite, and, less commonly, vaterite. These are distinct minerals with different physical properties, such as unit cell dimensions, effect on polarized light, stability, and even solubility. This last difference is the basis for distinguishing the different phases by staining techniques (Feigl, 1954). This approach is practical for large amounts of mineral such as is found in the molluscan shell, but it was found inappropriate for the calcium carbonate of the ligament and the powder method of X-ray diffraction was employed.

Stenzel (1962) found aragonite in the resilium of oysters and Bevelander and Nakahara (1969) reported that the inner ligament of *Mytilus edulis* and *Pinctada radiata* contained only long, needled shaped, single aragonite crystals. The ubiquity of aragonite in bivalve ligament, independent of the crystalline phase in the shell, suggests that certain of the physical properties of aragonite may be important to the mechanical operation of the ligament.

The Pectinidae which have the weakest ligaments also have the most resilient ligaments when based on the shell weight (Table III and Figure 4). Perhaps, the most complicated mechanical properties are observed in *Mytilus edulis* which appears to have the strongest abduction system to any of the species examined. The inner and outer ligaments are reduced; however, the periostracum of *Mytilus* is greatly elongated in the anteroposterior direction. Trueman (1960a) measured considerable extension and contraction of the periostracum. The elaborated development of and the mechanical properties of the periostracum may account for

the combination of resilience and strength. The mechanism by which this peculiar geometry affects strength and resilience is not apparent and needs further study.

The *Mytilus* abduction system is contrasted by *Ensis directus* and *Crassostrea virginica*, both of which are relatively weak abduction systems of low resilience (Table III and Figure 4). The burrowers are not outstanding in either strength or resilience. There is no clear trend relating life style to ligament strength or resilience in the species examined; however, it is notable that the swimmers are the most resilient and also the weakest.

In an attempt to relate the physical properties of the ligament with its structure and composition, the correlation coefficients were calculated for several sets of parameters as shown in Table IV. Only six of the experimental animals were included in the calculation; specimens of *Mercenaria mercenaria* and *Ensis directus* were excluded because these species have abducting external ligaments. Three sets of parameters show good correlation and resilience is involved in all three. This data is shown graphically in Figures 5, 6, and 7.

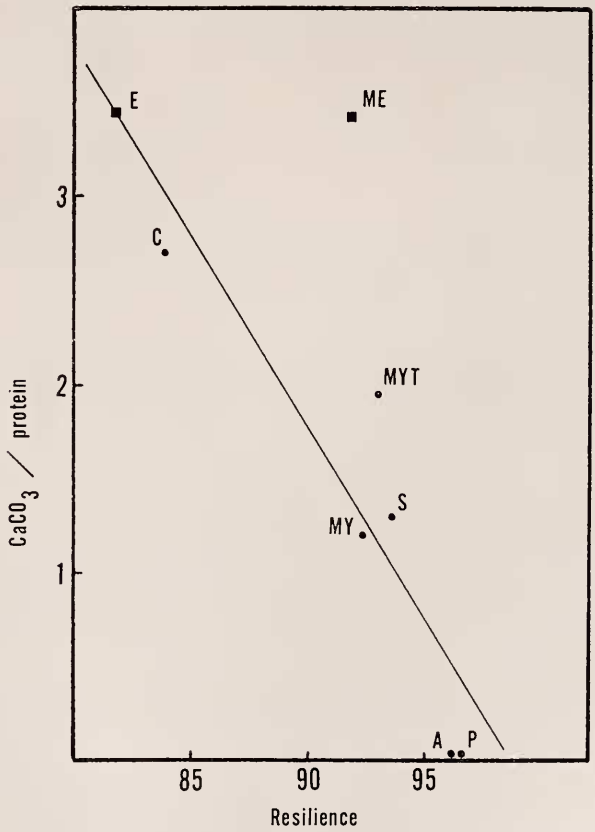

FIGURE 5. The $CaCO_3$/protein ratio as a function of resilience. Specimens of *Ensis* and *Mercenaria* were excluded in the calculation of the regression line—see text for explanation. Abbreviations as in Figure 4. The correlation coefficient is —0.88.

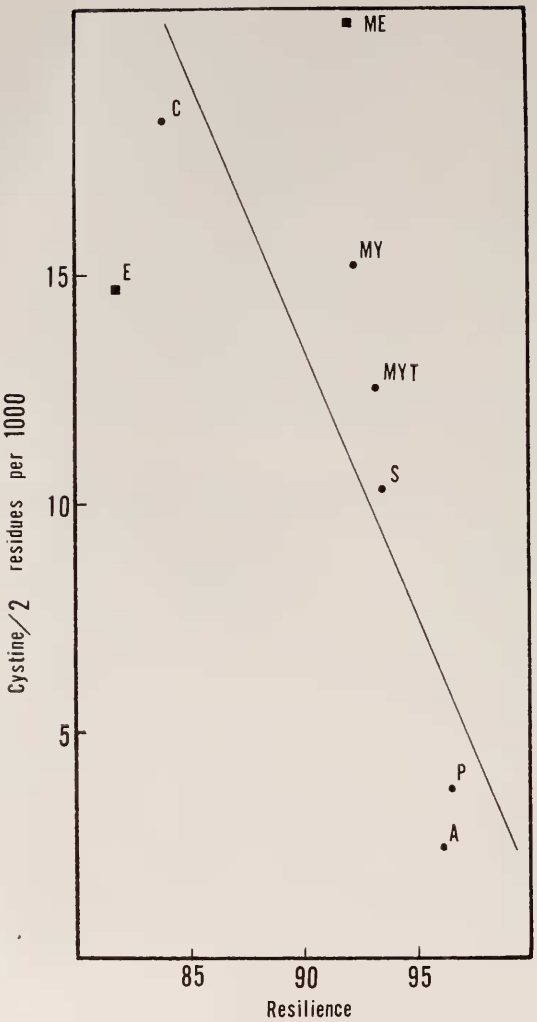

FIGURE 6. Cystine/2 residues per thousand as a function of resilience. Specimens of *Mercenaria* and *Ensis* were excluded in the calculation of the regression line—see text for explanation. Abbreviations as in Figure 4. The correlation coefficient is —0.86.

It appears that the aragonite crystals may interfere with the ability of the ligament to recover from compression (Figure 5). Similarly, larger concentrations of cystine/2 are correlated with lower resilience (Figure 6). This inverse relationship may be due to the stiffening of the ligament by the cross-linking of protein chains. Glycine concentration is directly related to resilience (Figure 7). Apparently the more closely the ligament approaches polyglycine, the more efficiently it can function. It is notable that the strength of the ligament (opening moment/g of shell) is not well correlated with any of the parameters considered in Table IV.

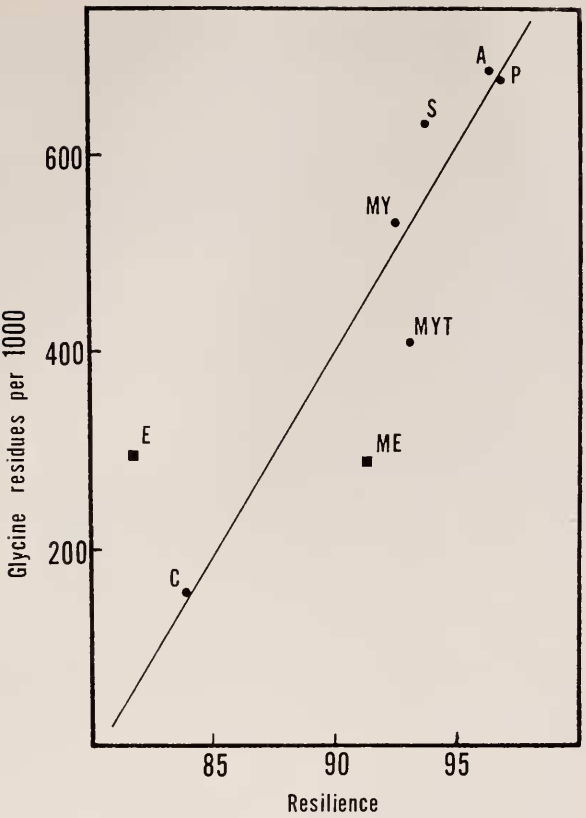

FIGURE 7. Glycine residues per thousand as a function of resilience. Specimens of *Mercenaria* and *Ensis* were excluded in the calculation of the regression line—see text for explanation. Abbreviations as in Figure 4. The correlation coefficient is 0.94.

The inverse relationship of cystine/2 and resilience, and the direct relationship of glycine concentration and resilience may support the theory of long thermally agitated protein chains acting with rubber-like elasticity. The greater the glycine concentration, the more the chains become rubber-like. Lower cystine/2 concentrations may indicate fewer cross-linkages and less restrictions on the chains, again suggesting a more rubber-like form. The possibility of β-type protein structure in the inner ligaments (as shown by X-ray diffraction) argues against rubber-like elasticity, as it requires that the chains be bound together in a backbone.

The ubiquity of the aragonite phase of $CaCO_3$ in ligaments is not readily explainable. It may be that the rod-like nature of aragonite, as opposed to calcitic spherules, provide a mechanical "firmness" similar to re-enforcing rods in concrete. It is notable that the essentially noncalcified ligaments of *Aequipecten* and *Placopecten* are among the weakest; however, the heavily calcified ligament of *Crassostrea virginica* is also relatively weak. The structural analysis of the inner ligament of *Spisula* will be considered in another publication.

We wish to thank the members of the Supply Department at the Marine Biological Laboratory for their prompt and efficient delivery of living material.

Summary

1. The bivalve ligament protein has a high percentage of glycine.

2. The absence of hydroxyproline and hydroxylysine in the ligament and the lack of a wide angle diffraction pattern indicate that the ligament is not collagen.

3. The ligament contains more sulfur amino acids—methionine and cystine/2—than other structural proteins.

4. Only the aragonite phase of calcium carbonate has been observed in association with the ligaments.

5. Members of the family Pectinidae have the weakest and most resilient ligaments.

6. Resilience is inversely correlated with $CaCO_3$ and cystine/2 concentration while glycine is directly correlated with resilience.

7. The strength factor (opening moment/gram of shell) is distinct from resilience and does not correlate with any of the parameters examined.

8. Recovery from compression by inner ligament is probably mediated through easing of steric strains induced during compression.

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